PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	
Briggs and Tatum	Group Art Unit: 1645
Serial No. 10/055,174	Examiner: J. Graser
Filed: January 25, 2002	Atty. Docket No. 000295.00014

For: ***LKTA* Deletion Mutant of *P. haemolytica***

DECLARATION OF ROBERT E. BRIGGS UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Robert E. Briggs, declare as follows:

1. I am named as an inventor in the application referenced above. My curriculum vitae is attached as Exhibit 1.

2. For the past 21 years I have studied bacterial respiratory disease of cattle and sheep years. In the past 12 years I have focused on molecular aspects of *P. haemolytica* (now known as *M. haemolytica*), *P. multocida*, and *H. somnus*, which are the principal bacterial etiologic agents involved in respiratory disease of cattle. These efforts resulted in techniques that for the first time made it possible to genetically engineer *M. haemolytica* and *H. somnus*. Using these techniques, we have produced genetically engineered products from each bacterium which have proven useful as attenuated live vaccines and for determination of the role played in

disease by important bacterial virulence factors. Modified-live products have proven to be effective mucosal vaccine candidates against shipping-fever of cattle both in laboratory and field trials. A number of products have been constructed which have demonstrated excellent efficacy in a multivalent vaccine used in sheep and goats, including an intranasally-delivered vaccine for pasteurellosis in bighorn sheep.

3. We conducted four field trials to evaluate oral or intranasal exposure to vaccines containing live lyophilized or previously lyophilized but reconstituted live leukotoxin-deficient *M. haemolytica* bacteria on the health and performance of feedlot cattle freshly placed into an experimental feedlot. In each trial, the leukotoxin-deficient *M. haemolytica* in the vaccine (D153ΔlktA34-378) do not express a biologically active leukotoxin, express a mutant leukotoxin protein that lacks amino acids 34-378, and contain no non-*M. haemolytica* DNA. The trials took place in the period from the Fall of 1998 through the Fall of 2003.

4. The first trial, conducted in the Fall of 1998, tested the efficacy of a vaccine containing reconstituted (formerly lyophilized) live bacteria administered by top-dressing on feed. This trial involved two groups of mixed breed beef calves weighing approximately 500 pounds each. One group (n=100) was procured predominantly from local sales barns by a reputable order-buyer in southwestern Arkansas (high-risk calves). The calves were collected at the order-buyer facility over a period of two days and appeared healthy and alert on the third day when they were processed. The calves were ear-tagged with sequential numbers. To yield an evenly balanced group of vaccinates and non-vaccinates, the odd-numbered calves were cut to a pen separate from even-numbered calves as they left the working chute. The second group (n=120) was collected concurrently from a single ranch in New Mexico, similarly ear-tagged, and cut to separate pens (low-risk calves).

5. The two groups, held at separate facilities about 800 miles apart, were given grass hay (25 pounds) and a pelleted starter ration (150 pounds) spread in 40 feet of feed bunk onto which the *M. haemolytica* vaccine was top-dressed. The vaccine consisted of approximately 50 grams of lyophilized powder containing approximately 10^9 CFU of bacteria/gram reconstituted in 1 liter Earle's Balanced Salt Solution, yielding a dose per calf of approximately 10^9 CFU / head (assuming all the feed is eaten and all the calves eat equally). The liquid was poured onto the feed in the bunks without further mixing into the feed. Non-vaccinated calves were similarly fed without top-dressed vaccine.

6. Vaccinates were held separately from non-vaccinates. Three days later, both groups of calves were trucked to an experimental feedyard in New Mexico (Clayton Livestock Research Center, New Mexico State University). Vaccinates were separated from non-vaccinates on separate decks of a semi-trailer (Arkansas calves) or on separate gooseneck trailers (ranch calves). On arrival at the feedyard, all calves received a clostridial vaccine product, Vitamins A, D, and E, pour-on wormer, and a modified-live IBR/PI-3 (bovine rhinotracheitis / parainfluenza 3) viral product. Nasal swabs and blood serum specimens were collected, and individual calf weights were recorded on the day of arrival and weekly thereafter for a period of 28 days.

7. Upon arrival at the feedyard, two low-risk calves were dead and one low-risk calf was moribund on the semi-trailer from Arkansas. All were non-vaccinates. Over the next three days, five additional non-vaccinated high-risk calves died, bringing the total mortality of this group to 8 calves (16%). Post-mortem showed cranioventral fibrinous pneumonia typical of pneumonic pasteurellosis. Culture results confirmed large numbers of *M. haemolytica*. In some calves, both *M. haemolytica* and *P. multocida* were present. In contrast, only two vaccinated

low-risk calves died (4%) over the course of the trial. One was a calf that exhibited an extended period of diarrhea and weight loss and died 10 days into the trial. This calf had lobular pneumonia, which yielded a pure culture of *P. multocida*. The other calf died near the end of the trial with multifocal pulmonary abscesses (typical of *Mycoplasma bovis*), and culture confirmed *M. bovis* infection. No death loss occurred among the low-risk calves, and their weight gain over the course of the trial exceeded that of non-vaccinates by 25%.

8. We conducted a second trial in the Fall of 2000 in which a lyophilized culture of the leukotoxin-deficient *M. haemolytica* was reconstituted and injected intranasally. This trial tested the effect of intranasal exposure to the live bacteria present in the reconstituted preparation on nasopharyngeal colonization by wild-type *M. haemolytica* in calves at the time of feedyard arrival. The trial involved 200 calves. Half the calves were purchased from an order buyer barn in Arkansas (AR calves; n = 100; mean body weight, 205 kg). The other calves were obtained from a single ranch in New Mexico (NM calves; n = 100; mean body weight, 188 kg). The calves were transported to a feedyard. At the time of arrival, a reconstituted preparation of the leukotoxin-deficient bacteria was administered intranasally to half of each group.

9. Calves were observed daily for respiratory tract disease (RTD). Nasal swab specimens were collected periodically to determine nasopharyngeal colonization status with *M. haemolytica*. Serum samples were assayed for antibodies to *M. haemolytica*. Fifteen AR calves had nasopharyngeal colonization by wild-type MH at the order buyer barn, whereas none of the NM calves had nasopharyngeal colonization. The intranasal exposure to the reconstituted, live leuktoxin-deficient bacteria elicited an increase in serum antibody titers against *M. haemolytica* in NM calves, but titers were less in NM calves treated for RTD.

10. These results demonstrate that exposure of NM calves to the reconstituted, live (previously lyophilized) leukotoxin-deficient bacteria offered protection from nasopharyngeal colonization by wild-type *M. haemolytica*. It is noteworthy that the intranasal vaccination was done after arrival at the feedyard, timing generally considered far too late to offer any benefit by conventional vaccination strategies. This trial is reported in Frank *et al.*, "Effect of intranasal exposure to leukotoxin-deficient Mannheimia haemolytica at the time of arrival at the feedyard on subsequent isolation of M haemolytica from nasal secretions of calves," Am J Vet Res. 2003 May;64(5):580-5 (Exhibit 4).

11. We conducted a third field trial in the fall of 2002 to evaluate a single-dose combination *Mannheimia haemolytica / Pasteurella multocida* edible modified-live vaccine formulation in beef calves. An additional experiment was superimposed within the trial to evaluate a commercial viral combination vaccine given twice before field exposure to calves known to be persistently infected with BVDV. Eighty-four approximately 225 kg mixed English breed beef calves 7-9 months of age were obtained by an order-buyer from a single cow-calf herd in southwestern Arkansas in mid-September. The calves were shipped to the order-buyer the afternoon prior to the day of processing.

12. On the day of processing, all calves were bled by jugular venipuncture for serum and EDTA specimens, wet and dry nasal swab specimens were collected, and rectal temperatures were recorded. Calves received consecutively numbered ear tags. Calves with odd-numbered tags received a modified-live combination viral vaccine product (Jencine 4, Schering-Plough) subcutaneously (left mid-cervical). Consecutive pairs of calves were cut to separate pens as they left the working chute to randomly assemble two groups of 42 calves each, balanced for virus vaccine treatment. These groups were held confined in separate pens overnight after processing,

with water but no feed and with no contact with other calves, to allow the calves to acclimate and increase their desire to eat.

13. The vaccine consisted of *M. haemolytica* strain D153ΔlktA34-378 and *P. multocida* strain P1062ΔhyaE. Late logarithmic growth was obtained in separate 100 ml Columbia broth cultures grown at 37°C with shaking. The cultures were combined and mixed with an equal volume (200 ml) 2X skim milk, which was then frozen at –80°C in a tray for lyophilization. After 4 days of lyophilization at 20°C shelf temperature, the material was crushed into powder and weighed. A portion representing 1 ml of the original culture was reconstituted and assayed by duplicate culture of serial dilutions. Colony counts indicated the 47 grams of recovered lyophilized powder contained 9×10^8 CFU *M. haemolytica* and 8.8×10^8 *P. multocida* per gram. The powder was stored in two ziplock bags, 20 grams each, and kept at –70°C or on dry ice until use. At the time of vaccination, one packet was mixed into 500 ml Earle's Balanced Salt Solution and then top-dressed onto feed in bunks sufficient to allow all calves to feed simultaneously.

14. The next day, about 36 hours after arrival, one group of calves received the oral vaccine preparation top-dressed onto 50 pounds of pelleted calf ration and ½ bale (12 kg) fresh grass hay. The control group of calves received feed similarly without vaccine. Most of the calves fed within 5 minutes of feed placement, and the bulk of the feed was consumed within 20 minutes.

15. Two days after vaccination the two groups of calves were placed into separate grass pastures. No contact was allowed between these two groups, and contact with other cattle was minimized to across-fence contact with few adult cattle. The goal was to minimize the

possibility that non-vaccinates might become exposed to the modified-live vaccine organisms likely to be shed to some degree by the vaccinates and to limit the possibility that wild-type *Mannheimia* or *Pasteurella* might be obtained from non-experimental calves. The calves were held under these conditions for 23 days, then loaded onto a semi-trailer for overnight shipment to the Clayton Livestock Research Center in Clayton, New Mexico. Calves were loaded onto separate decks of the trailer to further minimize the possibility of spreading the vaccine organism to control animals prior to sampling at the feedyard.

16. Eighteen additional head of calves were procured a few days before shipment from local auction markets by the order-buyer. These additional calves were included to supply additional and natural exposure to infectious agents by the principal calves, and were loaded on the fore and aft decks of the trailer to limit their contact with principal calves prior to initial sampling at the feedyard.

17. Upon arrival at the feedyard, the calves were weighed, specimens were collected as previously at the order-buyer facility. Virus-vaccinates were revaccinated, and all calves received wormer (Ivomec, Merial) and blackleg vaccine (Ultrachoice 7, Pfizer). The auction-market calves were ear tagged consecutively as they went through the working chute, as were eight additional calves previously determined to be persistently-infected with bovine viral diarrhea virus. Calves were sorted to six pens based on prior random allocation of ear-tag numbers, which mixed virus vaccine treatment and bacterial vaccine treatment as nearly as possible balanced for both treatment within each pen. Auction-market calves were randomly allocated three per pen to all six pens. Persistently-infected calves were non-randomly allocated two per pen to the first four pens. Pens were physically situated in a single row and were served

by separate feed bunks and waterers, but fences did allow nose to nose contact between adjacent pens.

18. Specimens were collected and body weights were recorded on days 0, 7, 14, and 35 after arrival at the feedyard. Sick animals were identified by pen riders based on a subjective clinical score for nasal and ocular discharge, respiratory rate and effort, body fill, lethargy, gait, and general appearance. Animals deemed clinically ill with undifferentiated respiratory disease were treated with Tilmicosin and penicillin as the primary antibiotics if their rectal temperature was 40 °C or greater. Retreatment was based on the same protocol.

19. At the order-buyer facility, 20 calves were treated for undifferentiated respiratory disease, and two required re-treatment (both non-vaccinates). One control calf died at the order-buyer due to a non-infectious injury sustained to its' cervical vertebrae as it interacted with the working chute during an effort to restrain it to treat undifferentiated respiratory disease. At the feedyard, control calves and bacterial vaccinated calves experienced similar overall pull rates at 32 (n=41) and 27 (n=42) pulls (potentially sick animals) respectively during the 35 day trial. Those which were febrile (and therefore were treated) were 20 and 19 respectively over the course of the trial. Calves which required multiple treatments for respiratory disease (retreatment) numbered 5 for non-vaccinates and 0 for vaccinates over the course of the trial. In comparison, auction-market calves experienced a total of 38 pulls (n=18) of which 22 required treatment, and the persistently infected calves experienced 12 pulls (n=8) of which 6 required treatment. Mortality was limited to two of the persistently infected calves.

20. Serum titers against *M. haemolytica* rose significantly in bacterial vaccinates as compared to control calves (Exhibit 2). Vaccinates' serum titers remained higher than controls, but not auction-market calves, throughout the 35 day trial at the feedyard. Auction-market

calves increased their serum titer, probably in response to natural infection, to closely match vaccinates within one week after arrival. Persistently infected calves did not respond to *M. haemolytica*, possibly because they were immunocompromised.

21. Vaccinated calves exhibited significantly greater weight gain ($p < 0.01$) during the first weeks of feeding compared to control calves and maintained their advantage through the 35 day trial (Exhibit 3). Vaccination improved average gain (kilograms of beef on the hoof gained after day 0) by 5.3 kg at 7 days on feed, 6.5 kg at 14 days, and 6.1 kg at day 35. Both groups of principals showed a gain advantage compared to auction-market calves, about 10.5 kg advantage ($p < 0.01$) among vaccinates and 4.5 kg among non-vaccinates (not significant) than auction-market calves. The principals would be expected to perform better than fresh auction-market calves due to the approximately 28 days of backgrounding which the principals but not the auction-market calves enjoyed.

22. We conducted a fourth trial in the fall of 2003 using a vaccine containing live, dry, lyophilized bacteria. The lyophilized bacterial vaccine was made two years prior to its use in the trial. It was not reconstituted, but was top-dressed dry onto feed. The virus revaccination was carried out at the order-buyer barn two weeks after the initial vaccination. Twelve calves received an experimental killed-virus preparation instead of a modified-live preparation. Otherwise, the methods were nearly identical to those used in the 2002 trial.

23. We are still conducting tests to analyze the results of this trial. Preliminary analysis, however, indicates that vaccination conferred an approximately 5 kilogram weight gain advantage ($p < 0.01$), as was observed in the 2002 trial. Sampling at arrival to the feedyard and after one week at the feedyard indicates that, despite commingling of vaccinates with non-

vaccinates, vaccination dramatically reduced nasal colonization by virulent *M. haemolytica* serotype 1 ($p < 0.001$).

24. Collectively, the four trials described above demonstrate that vaccination with the lyophilized or lyophilized and reconstituted vaccine strain:

- elicits a significant increase in anti-*Mannheimia* antibodies in calves after mucosal administration;
- reduces shedding and carriage of virulent *Mannheimia* in a herd (and therefore reduces the effective infectious load, an important predictor of risk);
- reduces the frequency of re-treatment for undifferentiated respiratory disease (which, according to some experts, is the most relevant indicator of calves which will suffer permanent performance reduction);
- reduces the mortality from pneumonic pasteurellosis; and
- increases the weight-gain of vaccinated calves (a very relevant measure of economic value to cattle producers).

25. I declare that all statements made herein of my own knowledge are true and that I believe all statements made on information and belief are true and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Mar-h 22, 2004
Date

Robert E. Briggs, D.V.M.

Exhibit 1

Dr. Robert E. Briggs
Curriculum Vitae

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Educational Background:

1975-1978	Iowa State University; Animal Sciences Pre-Vet
1978-1982	Iowa State University; DVM 1982
1982-1988	Iowa State University; MS 1988

Research Experience:

1982-1985	Postdoctoral Associate, Respiratory Disease of Cattle Research, USDA, ARS, NADC
1985-present	Veterinary Medical Officer, Respiratory Tract Diseases of Ruminants, USDA, ARS, NADC

Invitations in last 5 years:

Invited to National Cattlemens Beef Association Annual Convention in Charlotte NC, February, 1998, to present ongoing research on mucosal *P. haemolytica* vaccine.

Invited to Schering-Plough Animal Health, Elkhorn, NE, October 1998, to present ongoing research on oral vaccination of cattle.

Invited to Pfizer Central Research, Groton, CT, June, 1999, to present research findings on oral vaccination against shipping-fever.

Invited to Schering-Plough Animal Health, San Diego, CA, October 1999, to present ongoing research on mucosal vaccination strategies.

Invited to USAHA meeting in San Diego, CA, October 1999, to present results of research on mucosal *P. haemolytica* vaccine.

Invited to USAHA meeting in Birmingham, AL, October 2000, to present progress on mucosal *P. haemolytica* vaccine.

Invited to Diamond Animal Health/Agrilabs, St. Joseph MO, October 2001, to present research on vaccination strategies for cattle.

Invited to National Cattlemens Beef Association Annual Convention in Nashville TN, February, 2003, to present ongoing research on mucosal *P. haemolytica* vaccine.

Ad hoc reviewer for USDA NRI Competitive Grants Program, 1997-2003.

Referee for the journal "Vaccine", 1999, 2000.

External reviewer for Idaho Board of Education Research Center Grant Program, 1998.

Selected publications:

Sanders J. D.,Y. Tagawa, R. E. Briggs, and L. B. Corbeil. Transformation of a virulence associated gene of *Haemophilus somnus* into a strain lacking the gene. FEMS Microbiol. Letters. 154:251-258. 1997.

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Lehmkuhl H. D., R. E. Briggs, and R. C. Cutlip. Survey for antibodies to bovine adenoviruses in six- to nine-month-old feedyard cattle. Am J Vet Res 59:1579-1580. 1999.

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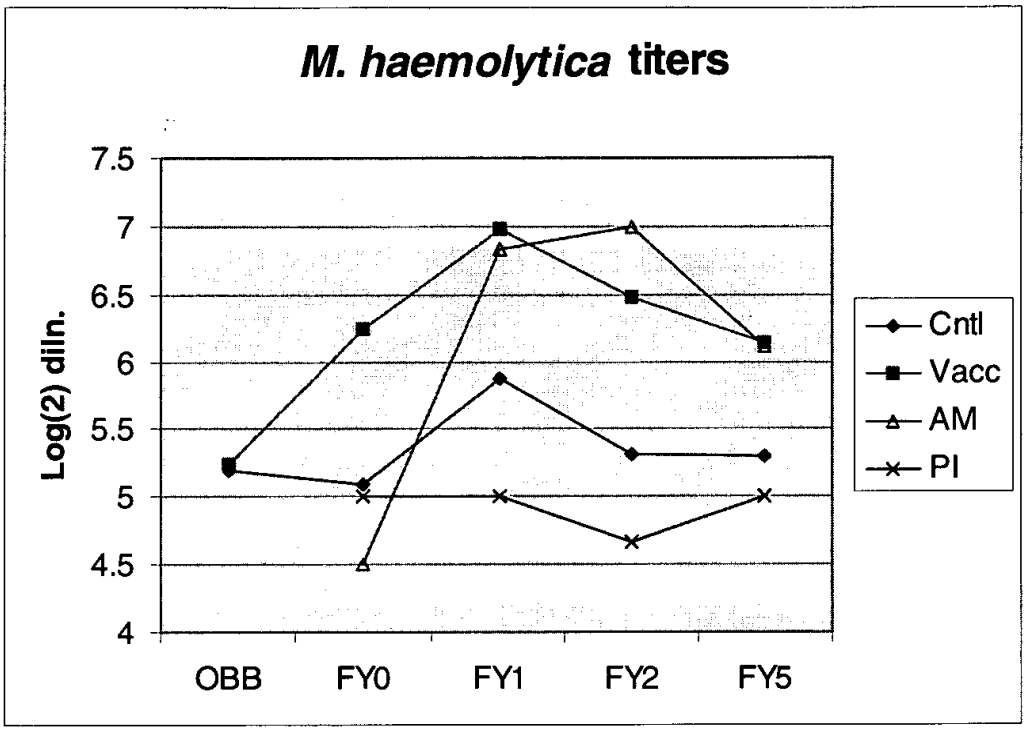
Exhibit 2



Cntl, control calves

Vacc, vaccinated calves

AM, auction-market calves

PI, persistently infected calves

OBB, order buyer barn

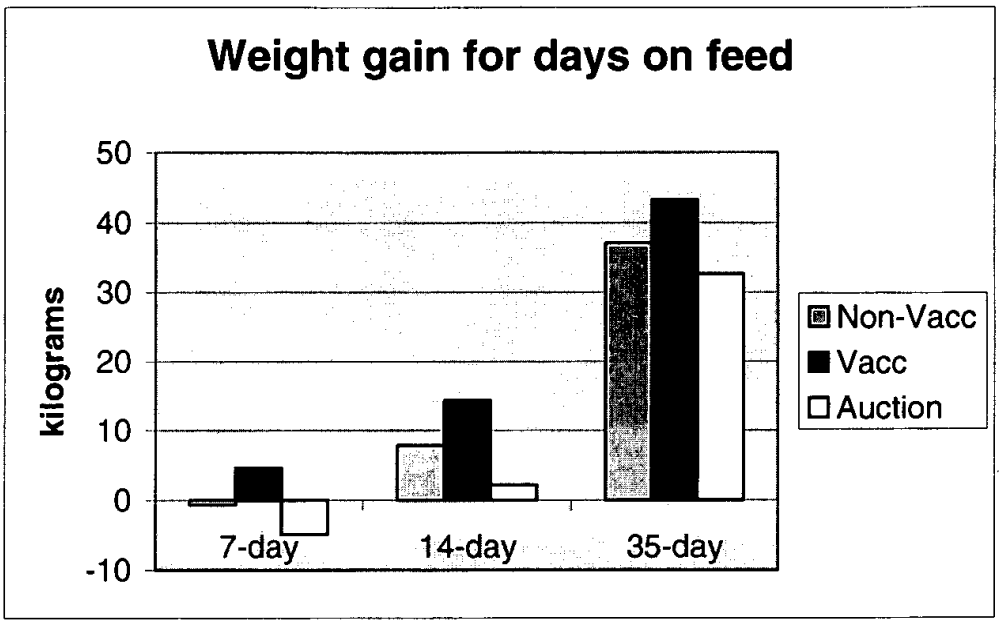
FY0, arrival at feed yard

FYI, week 1

FY2, week 2

FY5, week 5

Exhibit 3



Non-Vacc, non-vaccinated calves

Vacc, vaccinated calves

Auction, auction-market calves

Exhibit 4

Effect of intranasal exposure to leukotoxin-deficient *Mannheimia haemolytica* at the time of arrival at the feedyard on subsequent isolation of *M haemolytica* from nasal secretions of calves

Glynn H. Frank, DVM, PhD; Robert E. Briggs, DVM, MS; Glenn C. Duff, PhD; H. Scott Hurd, DVM, PhD

Objective—To determine the effect of intranasal exposure to live leukotoxin (LktA)-deficient *Mannheimia haemolytica* (MH) at the time of feedyard arrival on nasopharyngeal colonization by wild-type MH in calves.

Animals—200 calves.

Procedure—Calves from Arkansas (AR calves; n = 100; mean body weight, 205 kg) were purchased from an order buyer barn. Calves from New Mexico (NM calves; n = 100; mean body weight, 188 kg) were obtained from a single ranch. Calves were transported to a feedyard, where half of each group was exposed intranasally with LktA-deficient MH at the time of arrival. Calves were observed daily for respiratory tract disease (RTD), and nasal swab specimens were collected periodically to determine nasopharyngeal colonization status with MH. Serum samples were assayed for antibodies to MH.

Results—15 AR calves had nasopharyngeal colonization by wild-type MH at the order buyer barn, whereas none of the NM calves had nasopharyngeal colonization. Intranasal exposure to LktA-deficient MH elicited an increase in serum antibody titers against MH in NM calves, but titers were less in NM calves treated for RTD. Exposure of NM calves to LktA-deficient MH offered protection from nasopharyngeal colonization by wild-type MH.

Conclusions and Clinical Relevance—Exposure of calves to LktA-deficient MH elicited an increase in serum antibody titers against MH and decreased colonization of the nasopharynx by wild-type MH. Earlier exposure would likely allow an immune response to develop before transportation and offer protection from nasopharyngeal colonization and pneumonia caused by wild-type MH. (*Am J Vet Res* 2003;64:580–585)

Mannheimia haemolytica (MH) inhabits the tonsils and nasal passages of healthy cattle as a minor component of the normal bacterial flora.[1-3] After transport or during viral-induced illnesses, MH serotype A1 can undergo a rapid selective growth in the nasopharynx. This selective and substantial population increase is a likely prerequisite for the onset of pneumonic pasteurellosis.[4,7] An experimental parenterally administered MH bacterin inhibited colonization of the nasopharynx by MH.[4] In a previous experiment,[8] calves were vaccinated intranasally with a modified-live MH 3 days before transport to a feedyard, followed by prophylactic administration of antimicrobials at the time of arrival. The medication inhibited colonization by the vaccine strain of MH, and serum antibody titers against MH were decreased.[8] The study was designed to determine the effect of intranasal exposure of calves (at the time of arrival at the feedyard) to a live leukotoxin (LktA)-deficient MH on colonization of the nasopharynx by wild-type MH.

Received October 10, 2002.
Accepted December 13, 2002.

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The authors thank Jerold K. Peterson, Derek Walker, Kathryn Malcolm-Callis, Matt Wiseman, and Lisa Blan for technical assistance.

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Materials and Methods

Experimental design—Calves from Arkansas (AR calves; n = 100; body weight range, 175 to 241 kg; mean body weight, 205 kg; 54 bulls and 46 steers) were accumulated at a local order buyer barn on day –1. Calves were given sequential ear tag numbers as they were processed. Fifty calves (alternate sequentially numbered pairs) were vaccinated with a modified-live virus vaccine[a] that contained infectious bovine rhinotracheitis virus, bovine viral diarrhea virus types I and II, parainfluenza 3 virus, and bovine respiratory syncytial virus. All calves were transported by truck to the feedlot at the Clayton Livestock Research Center on day -1 and arrived on day 0. At the time of arrival, 50 odd-numbered calves (25 virus-vaccinated and 25 nonvaccinated) were exposed intranasally with live MH serotype 1 (exposed calves). The MH used for exposure had an approximate 1-kilobase deletion in the *lktA* gene encoding for leukotoxin (LktA-deficient MH).[9] This MH expresses and transports an inactive but immunogenic product, approximately 66 kDa molecular weight, from the partially-deleted *lktA* gene. The culture (3.5×10^7 colony-forming units/mL) was given intranasally (2 mL/nostril) with an automatic injector-drencher.[b] The nozzle of the injector-drencher was constricted to enhance projection of the effluent stream.

Calves from New Mexico (NM calves; n = 100; body weight range, 146 to 234 kg; mean body weight, 188 kg) originated from a single ranch. On day -1 at the ranch, calves were given sequential ear tag numbers as they were processed. Alternate sequentially numbered pairs were vaccinated with the modified-live virus vaccine. All calves were transported by truck to the feedlot at the Clayton Livestock Research Center on day 0 and arrived on day 0. At the time of arrival, 50 odd-numbered calves (25 virus-vaccinated, 25 nonvaccinated)

were exposed intranasally with the LktA-deficient MH by the same procedure that was used for AR calves.

At the time of arrival, all calves received SC vaccination with a clostridial antigen[c] and treatment for parasites.[d] Calves were weighed, branded, horn tipped, and received an injection[e] containing vitamins A and D_3. Bull calves were castrated. Calves were randomly assigned to 16 pens (12 to 13 calves/pen), within the constraints determined for each pen. Two pens contained exposed AR calves, 2 pens contained nonexposed AR calves, 2 pens contained exposed NM calves, 2 pens contained nonexposed NM calves, 4 pens contained exposed AR and NM calves (mixed pens), and 4 pens contained nonexposed AR and NM calves (mixed pens). All pens contained a balanced number of virus-vaccinates and nonvaccinates. Calves vaccinated with the modified-live virus vaccine were vaccinated with a modified-live bovine respiratory syncytial virus vaccine[f] on day 12.

Feed was provided for ad libitum consumption, and a continuous supply of water was available. Calves were monitored daily for clinical signs of respiratory tract disease (RTD). Clinical signs included nasal or ocular discharge, labored breathing, lethargy, and emaciated body condition. Calves that had clinical signs of RTD were taken to a processing facility, and those with a rectal temperature > 39.7°C received medical treatment. Calves were given tilmicosin[g] (10 mg/kg, SC) for the first treatment and florfenicol[h] (40 mg/kg, SC) and flunixin megulmine[i] (10 mg/kg, IM) for the second treatment, if necessary. A third treatment, enrofloxacin[j] (10 mg/kg, SC), was given if necessary. Additional treatments consisted of repeating the first 3 treatments. After each treatment, calves were returned to their pens.

All calves were weighed and rectal temperatures were recorded on days 0, 1, 6, 12, 19, and 33. Calves that had rectal temperatures > 39.7°C but had not been removed because of clinical signs of RTD were not treated. At the feedyard, pens of calves entered the chute for processing in an established sequence to control the spread of MH and viral exposure during processing. First to be processed were nonexposed NM calves from the nonmixed pens, then all nonexposed calves from the mixed pens, and then nonexposed AR calves from the nonmixed pens. The crowding pen, working alley, and squeeze chute were then disinfected with a spray.[k] After disinfection, exposed calves from nonmixed and mixed pens were processed in the same order as nonexposed calves, after which the handling equipment was again disinfected.

Nasal swab specimen and blood sample collection and bacteriologic culture—Nasal swab specimens were collected from all calves at the order buyer barn or ranch (day -1) and at the feedyard on days 1, 6, 12, 19, and 33. Blood samples were collected on the same days (except for day 1) to obtain serum samples. Nasal swab specimens were obtained by inserting 1 cotton-tipped swab into each ventral nasal meatus. Each pair of swabs was placed in a dry tube, sealed, and stored on dry ice for transport to the National Animal Disease Center, where they were stored at –70°C. For analysis, nasal swab specimens were applied to blood agar base[l] plates containing 5% bovine blood, using a constant procedure to form 3 zones of decreasing growth.[10] Zone 3 was an approximate 160-fold (mean, 161.5 ± 44.3-fold) dilution of zone 1. Cultures were incubated overnight at 37°C, and MH colonies were identified and serotyped.[11,12] The LktA-deficient MH was nonhemolytic, in contrast to the hemolytic wild-type. Serum antibody titers were determined by use of an indirect hemagglutination procedure.[13] A calf was considered to have nasopharyngeal colonization by MH if the nasal swab specimen had positive bacteriologic culture results for MH.

Statistical analyses—The effect of LktA-deficient MH exposure on isolation of wild-type MH and on the proportion of calves treated was evaluated by χ^2 and logistic regression analyses. The proportion of calves treated anytime, on day 2 or later, and on day 6 or later was evaluated. Potential confounding variables analyzed in the logistic model included source of calves, pen identification number, vaccine virus administration, and whether they were in a mixed pen. Results from the most parsimonious model are presented for the 3 dependent variables. A value of $P < 0.05$ was considered significant.

Results

Effects of exposure on RTD—No deaths occurred as a result of RTD. Most calves (73.5%; 147/200) were treated on at least 1 day. Sixty-two NM calves and 85 AR calves were treated on at least 1 day with antimicrobials (Table 1). All but 24 treatment days occurred on day 6 of feed or earlier. More AR calves than NM calves were treated on day 6 or earlier (Fig 1). Calves that were exposed to LktA-deficient MH were significantly less likely to be treated after day 6 on feed, compared with nonexposed calves (37.5% [9/24] vs 62.5% [15/24], respectively). This effect was most notable in NM calves. Exposure had no effect on the proportion of calves treated on day 6 of feed or earlier. Findings on logistic regression models revealed that the exposure to LktA-deficient MH decreased the probability of treatment on day 6 or later, but not on day 2 or later (odds ratio, 0.43). The model included the covariates of source (New Mexico or Arkansas), whether calves were from a mixed pen, and whether calves had received the virus vaccine.

Colonization by MH—Colonization of the nasopharynx by wild-type MH was influenced by origin, exposure to LktA-deficient MH, and whether calves were kept in a mixed pen. Fifteen AR calves had colonization by wild-type MH at the order buyer barn, whereas none of the NM calves had colonization by wild-type MH at the ranch (Table 2 and 3; Fig 1). On the first day after arrival (day 1), 43 to 83% of the AR calves from each mixed pen and 50 to 77% of the AR calves from each nonmixed pen had nasopharyngeal colonization by wild-type MH. The infection spread to NM calves from mixed pens and peaked at day 12. Wild-type MH was recovered from nonexposed NM calves from mixed pens by day 6,

Table 1—Calves treated for respiratory tract disease with antimicrobials

Source	Exposed*	Mixed†	Calves: Total No.	Calves: Total No. treated
Arkansas	Yes	Yes	24	21
Arkansas	Yes	No	28	24
Arkansas	No	Yes	26	22
Arkansas	No	No	24	18
New Mexico	Yes	Yes	26	19
New Mexico	Yes	No	24	18
New Mexico	No	Yes	24	17
New Mexico	No	No	26	8

*Exposed intranasally to live *Mannheimia haemolytica* serotype 1 with a deletion in the *lktA* gene encoding for leukotoxin at the time of arrival at a feedyard in New Mexico. †Held in same pen with calves from the other source.

but was not recovered from exposed NM calves from mixed pens until day 12. Wild-type MH was not recovered from nonexposed NM calves from nonmixed pens until day 12. Isolation rates seemed to peak around day 33, but no data are available beyond day 33. One day after intranasal exposure to LktA-deficient MH, wild-type MH was isolated from 51 AR calves (22 exposed and 29 nonexposed), whereas the LktA-deficient MH was isolated from 23 of 50 exposed AR calves. The LktA-deficient MH was isolated from 26 of 50 exposed NM calves on day 1 and from 13 of 50 exposed NM calves on day 6. None of the nonexposed calves had nasopharyngeal colonization by LktA-deficient MH during the trial. Given that NM calves were exposed at the feedyard and AR calves seemed to bring infection to the feedyard, NM calves could be viewed as the study population for determination of the effect of LktA-deficient MH. Isolation of wild-type MH on day 6 or later was significantly lower in all exposed calves, compared with nonexposed calves (30.9% [29/94] vs 69.2% [65/94], respectively). No wild-type MH was isolated from exposed NM calves from nonmixed pens. Wild-type MH was isolated from only 42% (21/50) of exposed NM calves from mixed pens. On the basis of the logistic regression model, the odds of recovering wild-type MH were 5-fold less for exposed calves than nonexposed.

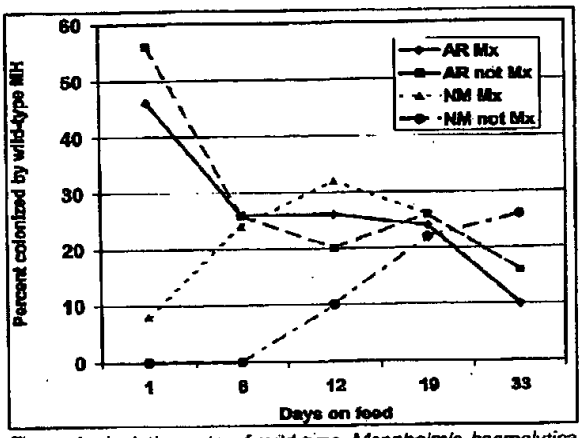

Figure 1—Isolation rate of wild-type *Mannheimia haemolytica* (MH) by source and pen (mixed or nonmixed). Calves were randomly assigned to 16 pens, with 12 to 13 calves in each. Four pens contained only calves from Arkansas (AR calves), and 4 contained only calves from New Mexico (NM calves). Four pens contained exposed AR calves and exposed NM calves (mixed pens), and 4 contained nonexposed AR calves and nonexposed NM calves (mixed pens). Mx = Mixed pen.

Table 2—Effects at the time of feedyard arrival of intranasal exposure of calves from Arkansas with LktA-deficient *Mannheimia haemolytica* (MH) and of placement in pens with calves from New Mexico (mixed pens) on the recovery of wild-type and LktA-deficient MH from nasal swab specimens

Exposed	Mixed pen	Day of study	No. of calves	No. of calves with nasopharyngeal colonization: Wild-type MH	No. of calves with nasopharyngeal colonization: LktA-deficient MH
Yes	Yes	–1	24	5	0
Yes	No	–1	26	3	0
No	Yes	–1	26	3	0
No	No	–1	24	4	0
Yes	Yes	1	24	8	11*
Yes	No	1	26	14	12*
No	Yes	1	26	15	0
No	No	1	24	14	0
Yes	Yes	6	24	6	3
Yes	No	6	26	4	0
No	Yes	6	26	7	0
No	No	6	24	8	0
Yes	Yes	12	24	6	2
Yes	No	12	26	3	0
No	Yes	12	26	7	0
No	No	12	24	7	0
Yes	Yes	19	24	6	2
Yes	No	19	26	4	0
No	Yes	19	26	6	0
No	No	19	23	9	0
Yes	Yes	33	24	3	0
Yes	No	33	26	1	0
No	Yes	33	26	2	0
No	No	33	23	7	0

*Significantly ($P < 0.05$) greater than number of nonexposed calves.

Effects of antimicrobial treatment on MH isolation—The first treatments for RTD were administered on day 1. On day 6, MH was isolated from 19 of 81 treat-

Table 3—Effects at the time of feedyard arrival of intranasal exposure of calves from New Mexico with LktA-deficient MH and of placement in pens with calves from Arkansas (mixed pens) on the recovery of wild-type and LktA-deficient MH from nasal swab specimens

Exposed	Mixed pen	Day of study	No. of calves	No. of calves with nasopharyngeal colonization: Wild-type MH	No. of calves with nasopharyngeal colonization: LktA-deficient MH
Yes	Yes	–1	26	0	0
Yes	No	–1	24	0	0
No	Yes	–1	24	0	0
No	No	–1	26	0	0
Yes	Yes	1	26	0	16*
Yes	No	1	24	0	10*
No	Yes	1	24	4	0
No	No	1	26	0	0
Yes	Yes	6	26	5	7*
Yes	No	6	24	0	6*
No	Yes	6	24	7†	0
No	No	6	26	0	0
Yes	Yes	12	26	5	3
Yes	No	12	24	0	3
No	Yes	12	24	11‡	0
No	No	12	26	5	0
Yes	Yes	19	26	5	0
Yes	No	19	24	0	1
No	Yes	19	24	8‡	0
No	No	19	26	11§	0
Yes	Yes	33	26	2	0
Yes	No	33	24	0	1
No	Yes	33	24	6‡	0
No	No	33	26	13§¶	0

*Significantly ($P < 0.05$) greater than number of nonexposed calves. †Significantly ($P < 0.05$) greater than number of calves from nonmixed pens. ‡Significantly ($P < 0.05$) greater than number of exposed calves from nonmixed pens. §Significantly ($P < 0.05$) greater than number of exposed calves. ¶Significantly ($P < 0.05$) greater than number of exposed calves from mixed pens.

Table 4—Isolation of all MH (deletion mutant and wild-type) from nasal swab specimens of calves treated and untreated for respiratory tract disease within 6 days prior to specimen collection

Source	Day of study	No. of calves* Treated	No. of calves* Not treated
Arkansas	6	19/81	8/19
Arkansas	12	0/21	25/79†
Arkansas	19	0/13	25/87
Arkansas	33	0/0	13/100
New Mexico	6	4/41	20/59†
New Mexico	12	0/5	25/95
New Mexico	19	0/18	23/82†
New Mexico	33	0/0	22/100

*Total number of calves from which MH was isolated over number of calves that were treated or not treated. †MH isolated from significantly ($P < 0.05$) more untreated calves than treated calves.

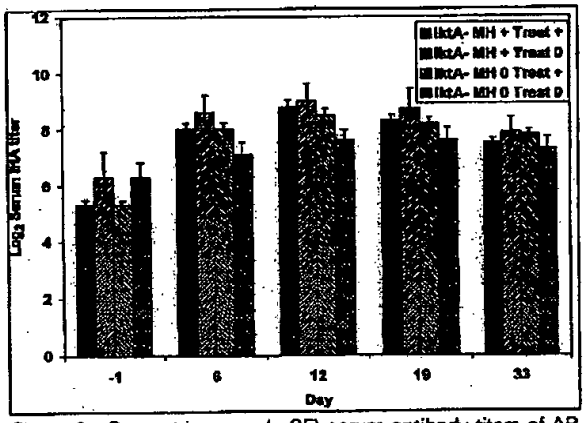

Figure 2—Geometric mean (± SE) serum antibody titers of AR calves against MH as determined by means of an indirect hemagglutination (IHA) inhibition assay. IktA-MH + = Calf exposed intranasally at the time of feedyard arrival to five MH serotype 1 that had a deletion in the *lktA* gene encoding for leukotoxin. IktA-MH 0 = Calf not exposed. Treat + = Calf treated for respiratory tract disease during days 1 to 5 of study. Treat 0 = Calf not treated during days 1 to 5.

ed calves and from 8 of 19 calves that were not treated during days 1 to 5. On days 12, 19, and 33, MH was not isolated from 34 nasal swab specimens of calves that were treated within 7 days before sample collection, but MH was isolated from 66 of 266 nasal swab specimens of untreated calves (Table 4). Treatment for RTD during days 1 to 5 significantly decreased the number of NM calves from which MH was isolated on day 6.

Serotypes of MH isolates—Two serotypes of wild-type MH, A1 and A2, were isolated from bacteriologic cultures of nasal swab specimens. At the order buyer barn, 11 of 26 isolates from AR calves were serotype A2, whereas no MH was isolated from NM calves at the ranch. In the feedyard, serotype A1 comprised 123 of 134 (93%) of all wild-type MH isolates from AR calves and 81 of 93 (87%) of the wild-type isolates from NM calves. Most of the wild-type serotype A1 MH colonies tested were resistant to kanamycin.[8]

Serum antibody titers against MH—Serum antibody titers against MH were determined by an indirect hemagglutination procedure. Serum antibody titers of AR calves increased by day 6, but serum antibody titers

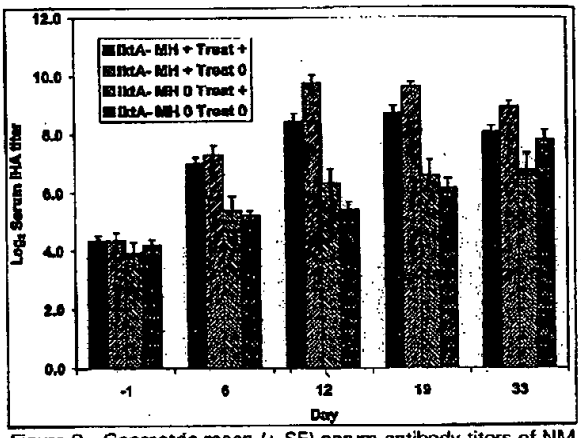

Figure 3—Geometric mean (± SE) serum antibody titers of NM calves against MH as determined by means of an IHA inhibition assay. *See* Figure 2 for remainder of key.

against MH between exposed and nonexposed calves were not significantly different (Fig 2). Treatment for RTD during days 1 to 5 did not inhibit the development of serum antibody titers against MH by day 6. However, serum antibody titers against MH of nonexposed AR calves that were not treated were significantly lower than those of the other groups on days 6 and 12.

Serum antibody titers against MH of NM calves increased by day 6, and serum antibody titers of exposed NM calves were significantly greater than those of nonexposed calves through day 19 (Fig 3). From days 12 to 33, exposed NM calves that were treated for RTD during days 1 to 5 had significantly lower serum antibody titers against MH than those of untreated calves. Serum antibody titers of nonexposed NM calves that were not treated were significantly lower than those of the other groups on day 12 but were significantly greater than nonexposed treated calves by day 33. Nonexposed NM calves from mixed pens developed greater serum antibody titers against MH than those of nonexposed NM calves from nonmixed pens through day 19. By day 33, serum antibody titers of nonexposed NM calves were no longer significantly different between calves from mixed and nonmixed pens.

Discussion

Acute RTD, in the form of an acute pneumonic pasteurellosis, usually develops in transported calves within the first week of arrival at the feedyard and was found in the calves of our study. *Mannheimia haemolytica* inhabits the tonsils and nasal passages of healthy cattle as a minor component of the normal bacterial flora.[1-5] After transport, MH serotype A1 can undergo rapid selective growth in the nasopharynx,[6,7] which was also found in the calves of our study. In our study, 2 groups of calves (1 from Arkansas and 1 from New Mexico) were brought to a common feedyard, where half of each group was placed in pens with calves from the other source and half of each group was not. At least 15 AR calves had colonization of the nasopharynx by MH serotype A1 before transport, and 51 AR calves had colonization on day 1 after transport. However, NM calves appeared to be free of MH colonization, because

none had positive bacteriologic culture results for wild-type MH before transport or on day 1. This presented an opportunity to study the effect of exposure to LktA-deficient MH on wild-type MH colonization of calves with and without preexisting MH infections.

Spread of wild-type MH infection was influenced by preexisting MH infections, exposure to LktA-deficient MH, placement of uninfected calves in pens with calves that had colonization of the nasopharynx by MH, and treatment for RTD. Because many of the AR calves already had colonization of the nasopharynx by wild-type MH, results of the LktA-deficient MH exposure were more evident in the NM calves. Exposure to LktA-deficient MH at the time of arrival inhibited colonization by wild-type MH in NM calves from nonmixed pens, whereas some nonexposed calves had colonization of the nasopharynx by MH within 12 days, even though they had not been placed in pens with AR calves.

Tilmicosin and florfenicol are used to treat RTD. Both antimicrobials inhibit colonization of the nasopharynx by MH for several days after administration.[8,10,14] In our study, treatment did not significantly decrease the number of AR calves with detectable colonization on day 6. However, most treated calves were shedding less MH organisms than untreated calves. With the procedure used to streak the agar plates to create 3 zones of a decreasing number of colonies, zone 3 is an approximate 160-fold dilution of zone 1. Of the 19 treated calves from which MH was isolated on day 6, bacteriologic culture results of nasal swab specimens revealed that 4 calves had only 1 detectable MH colony in zone 1, 6 calves had multiple MH colonies in zone 1 only, 7 calves had MH colonies in zone 2, and 2 calves had MH colonies in zone 3. Of the 8 untreated calves from which MH was isolated on day 6, bacteriologic culture results of nasal swab specimens revealed that 1 calf had MH colonies only in zone 1, 2 calves had MH colonies in zone 2, and 5 calves had MH colonies in zone 3. The number of untreated calves with MH colonies in zone 3 was significantly greater than that of treated calves.

In the AR calves, serum antibody titers against MH increased by day 6 with no significant differences in geometric mean titer between exposed and nonexposed calves. Serum antibody titers against MH of untreated nonexposed calves were less than those of the other groups. Serum antibody titers against MH of exposed untreated calves were greater than those of nonexposed untreated calves, supporting the probability that exposure elicited an increase in serum antibody titer. In the NM calves, exposure elicited significantly higher serum antibody titers against MH, compared with nonexposed NM calves.

Because a dramatic selective increase in the MH population of the nasopharynx is a prerequisite for the development of pneumonic pasteurellosis, inhibition of colonization of the nasopharynx of calves by MH is a desirable goal to seek in order to control the disease. One method of inhibition was the prophylactic use of tilmicosin or florfenicol before transport or at the time of arrival at the feedyard.[8,14] Another was by vaccination with an experimental MH serotype A1 bacterin made by growing the bacteria in the presence of bovine lung lavage and spleen cells.[1] The MH organisms grown in bovine cellular material may produce some of the same antigens that they would produce in vivo. Exposure to live attenuated MH intranasally would allow the bacteria to produce the products necessary for survival in the host and the host to produce antibodies against these products. In our study, calves were exposed to live attenuated MH that colonized the nasopharynx. Even though the exposure was not given early enough to allow immune responses to develop before contact with wild-type MH, an antibody response to the attenuated MH and some protection against colonization by wild-type MH were observed.

One drawback to using live bacteria as a vaccine for RTD at the time of arrival at the feedyard is that antimicrobial treatment for RTD would inhibit its growth in the host and result in a decrease in the antibody response.[8,14] In our study, the first treatments for RTD were given 1 day after arrival at the feedyard, which was 1 day after exposure. Treatment caused the development of serum antibody titers to be decreased in exposed and nonexposed NM calves. Presumably, the decrease in the development of serum antibody titers against MH was caused by suppression of natural MH infection by the treatment.

In our study, intranasal exposure of calves to LktA-deficient MH at the time of arrival at the feedyard increased serum antibody titers to MH and decreased the amount of colonization of the nasopharynx by wild-type MH. Therefore, it would seem that exposing calves to LktA-deficient MH at an earlier time to allow an immune response to develop before calves are stressed by transport to a feedyard could offer protection from colonization and from pneumonia caused by wild-type MH.

[a]Titanium 5, Agri Laboratories Ltd, St Joseph, Mo.
[b]Alliflex, NJ Phillips Pty Ltd, Somersby, NSW 2250, Australia.
[c]Electroid 7, Schering-Plough Animal Health Corp, Omaha, Neb.
[d]Cydectin, Fort Dodge Animal Health, Fort Dodge, Iowa.
[e]Vita-Jec A and D, RXV Products, Grapevine, Tex.
[f]Titanium BRSV, Agri Laboratories Ltd, St Joseph, Mo.
[g]Micotil, Elanco Animal Health, Indianapolis, Ind.
[h]Nuflor, Schering-Plough Corp, Kenilworth, NJ.
[i]Banamine, Schering-Plough Corp, Kenilworth, NJ.
[j]Baytril, Bayer Corp, Shawnee Mission, Kan.
[k]Lysol IC, Racket and Coleman Inc, Montvale, NJ.
[l]Bacto blood agar base, Difco Laboratories Inc, Detroit, Mich.
[m]Kanamycin A, Sigma Chemical Co, St Louis, Mo.

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